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Studies on the Immunogenicity of Synthetic and Natural Thomsen-Friedenreich (TF) Antigens in Mice: Augmentation of the Response by Quil A and SAF-m Adjuvants and Analysis of the Specificity of the Responses

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ABSTRACT

Galβ1-3GalNAcα-O-Serine/threonine (TF antigen) is expressed in the mucins of most epithelial cancers. We have compared the effect of five immunologic adjuvants on the immunogenicity in mice of TF antigen naturally expressed on porcine submaxillary mucin (PSM) with synthetic TF disaccharide covalently attached to ceramide or keyhole limpet hemocyanin (KLH). Natural and synthetic TF antigen expressed on PSM and TF ceramide, respectively, were minimally immunogenic, and antibodies were not significantly augmented by any of the immunologic adjuvants tested. TF disaccharide-KLH was moderately immunogenic, and antibody titers could be augmented greatly with the use of the immunologic adjuvants QS21 (containing a purified Quil A saponin fraction) and SAF-m (containing threonyl MDP and pleuronic L121 block copolymer). The low level natural IgM antibody titers against TF disaccharide-HSA were augmented by these immune procedures, and these higher titer IgM antibodies reacted as well with TF antigen naturally expressed on tumor mucins or desialylated red cell glycoproteins. High titer IgG antibodies induced against the TF disaccharide conjugate, however, failed to react with naturally occurring TF antigen from either source. IgG antibody titers against other epitopes on PSM or KLH also were highest with the use of QS21 and SAF-m. Our studies identify covalent attachment of TF disaccharide to KLH as an approach capable of consistently augmenting IgG antibody titers against this synthetic disaccharide, and the immunologic adjuvants QS21 and SAF-m as optimal for inducing anti-TF disaccharide, anti-PSM and anti-KLH IgG antibodies. They also suggest that in the presence of T cell help (as provided by KLH), the TF disaccharide epitope recognized by B cells is different from that recognized in the absence of T cell help.

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synthesized and used with a 2-carbon (crotyl) linker arm to prepare TF-KLH and TF-HSA by Biomira Inc. as previously described.[7] Keyhole limpet hemocyanin (KLH) was purchased from Cal-Biochem (La Jolla, CA), and human serum albumin (HSA) was purchased from Sigma Chemical Co. (St. Louis, MO). Tn antigen (GalNAcα-) covalently attached to HSA (Tn-HSA) and sialosyl Tn-HSA were prepared from synthetic crotyl-Tn (or sialosyl Tn) preparations as described for TF-KLH and TF-HSA. Epiglycanin was purified from ascites of BALB/c mice bearing TA3-HA IP tumors, as previously described.[11] Glycophorin was purified from human RBC and desialylated as described previously by Springer and Desai.[12]

Immunologic adjuvants

Salmonella minnesota mutant R595 was prepared as previously described[13] DETOX containing monophosphoryl lipid A (MPLA) and bacille Calmette-Guérin cell wall skeleton (BCGCWS) was purchased from Ribi Immunochem (Hamilton, MT), and complete Freund's adjuvant (CFA) was purchased from Difco Laboratories (Detroit, MI). SAF-m, obtained from Syntex Research (Palo Alto, CA), contains threonyl MDP, squalane, pleuronic L121 block polymer, and Tween 80 in phosphate-buffered saline.[14] QS21 containing a purified Quil A saponin fraction was obtained from Cambridge Bioscience (Worcester, MA).[15]

Vaccine preparation

Fifty micrograms of PSM or 25 μg of TF disaccharide-ceramide or TF-KLH were mixed with PBS, DETOX (2.5/25 μg MPLA/BCGCWS), SAF-m (50 μg), or QS21 (10 μg). These doses of PSM and TF-KLH (3100:1) and TF-ceramide were selected to provide approximately 12 μg of TF disaccharide per vaccine. TF-ceramide also was adsorbed to R595 cells or emulsified in complete Freund's adjuvant, as previously described.[13] Proteosomes were prepared from 25 μg TF-ceramide or TF-KLH, plus 12.5 μg outer membrane proteins obtained from *Neisseria meningitidis* as previously described.[16]

Vaccine administration

In each experiment, five mice, selected randomly from the same shipment, were immunized three times with a given vaccine. The three vaccinations were administered 2–3 weeks apart SC in a total volume of 0.1 mL/mouse. CFA, and to a lesser extent SAF-m, resulted in palpable and sometimes crusting lesions at vaccination sites. No other morbidity was detected. This vaccination schedule, including pretreatment with Cy, is based on our previous studies with gangliosides[13] and has not been varied with these antigens.

Serologic assays

Mice were bled from the retroorbital sinus before and 2 weeks after each vaccination, and serum samples for serologic testing (approximately 0.1 mL) were stored at −20°C. Murine monoclonal antibodies B72.3 (IgG) and 49H.8 (IgM) were used as positive control antibodies against sialylated Tn[17] and TF antigen.[18] ELISA was performed using rabbit antimouse IgM and IgG and protein A, linked to alkaline phosphatase (Zymed, San Francisco, CA) as previously described.[19] The absorbance of samples tested on HSA alone was subtracted from the absorbance tested on TF-HSA to yield the experimental values at each titer. Serologic titer in ELISA is defined as the highest dilution yielding a corrected absorbance of 0.10 or greater. All assays were repeated on two or more occasions to ensure consistency. Dot blot immune stain assays were performed as previously described,[20,21] with slight modifications. In brief, 0.5 μg of the various antigens were spotted on nitrocellulose paper strips. The strips were then blocked at room temperature for 2 h in phosphate-buffered saline containing 3% BSA. Serum was diluted 1:150 with the same buffer, and the strips were incubated in Accutron trays (Schleicher and Schull, Keene, NH) at room temperature for 16 h. Strips were then washed five times with the phosphate-buffered saline mixture and incubated for 5 h with horseradish-peroxidase-conjugated anti-(mouse IgM or IgG) antibody diluted 1:150 (Zymed, San Francisco, CA, or Southern

Biotech, Birmingham, AL, respectively). Peroxidase staining was performed as previously described.[19] The staining intensity of each spot was designated as negative, 1+, 2+, or 3+, as shown in Figures 2 and 3.

Delayed type hypersensitivity (DTH)

Two weeks after the first, second, or third immunization, 5 μg TF-HSA and HSA were injected in 0.05 mL PBS into the right and left hind footpads, respectively. Footpad thickness before and after DTH testing at intervals of 24 and 48 h were measured and compared.

RESULTS

Serologic response against TF antigen after vaccination

Preimmunization sera showed low titer IgM reactivity with TF disaccharide, PSM, and epiglycanin and low titer IgG reactivity with epiglycanin (Table 1, Fig. 1). Immunization with synthetic TF disaccharide-ceramide or porcine submaxillary mucin (PSM) alone did not induce an antibody response. Mixed with various adjuvants, TF disaccharide-ceramide and PSM induced low titer IgM and IgG responses against TF-HSA in occasional mice (Table 1). High titer IgG responses against PSM were seen in mice immunized with vaccines containing PSM plus SAF-m or QS21, but no significant reactivity against epiglycanin or asialoglycophorin was induced (results not shown). No reactivity was seen on completely deglycosylated PSM, suggesting that reactivity was not against the PSM protein backbone or that this protein was denatured by the dehydration procedure, and so the antigenic epitopes recognized by these anti-PSM antibodies remain unknown. TF disaccharide was significantly more immunogenic when linked to KLH, especially at the higher epitope ratio (3100:1), inducing IgM and IgG antibodies in most mice. Complete Freund's adjuvant, Detox, and proteosomes had little impact on this level of reactivity. SAF-m and QS21, however, resulted in significant further augmentation of the IgM antibody response (from a median titer of 1/20 to 1/60) and the IgG antibody response (from 0 to >1/5000). The range of IgG antibody titers against TF-HSA in the 40 mice immunized with TF-KLH plus SAF-m or QS21 was 1/1280–1/36,450. The range in the 35 mice immunized with TF-KLH plus Detox, CFA, or proteosomes was 0–1/1280. The impact of QS21 and SAF-m on IgG ELISA reactivity against TF disaccharide and PSM compared to CFA or no adjuvant is shown in Figure 1.

Specificity of TF antigen-reactive sera defined by dot blot immune stains

The specificity of all high titer IgM and IgG antisera (greater than 1/160 was studied. A sample dot blot immune stain experiment with sera obtained before and after immunization is shown in Figure 2. The increased IgM reactivity for asialoglycophorin and epiglycanin after immunization indicates that the immunogenic epitope on synthetic TF disaccharide conjugates is able to boost natural IgM antibody titers against natural TF antigen. Other antigens tested (but not shown) include glycophorin (negative) and ovarian carcinoma mucin (strongly positive), reinforcing the conclusion that these IgM antibodies react with natural TF antigen. On the other hand, Figure 3 shows the lack of consistent reactivity of IgG antibodies with asialoglycophorin and epiglycanin (beyond the 1+ natural antibodies against epiglycanin seen in most mice before and after immunization). This result indicates that the high titer IgG response is against an epitope(s) on synthetic TF disaccharide conjugate, which is not present on natural glycoproteins containing TF antigen, and no reactivity was seen with ovarian carcinoma mucin (results not shown). The origin of the anti-HSA reactivity seen in QS21 and SAF-m treated mice (Fig. 3) is assumed to be epitopes on KLH cross-reactive with HSA, since HSA reactivity was not seen after vaccination with PSM or TC plus SAF-m or QS21, and these mice had not received TF-HSA footpad injections for DTH. When IgG dot blot immune stains were performed at a serum dilution of 1:2000 instead of 1:150, the only reactivity remaining was with TF-HSA in all SAF-m and QS21, and occasional Detox, treated mice. This dot blot reactivity generally could still be detected at a serum dilution of 10^6.

TABLE I. PEAK TITER SEROLOGIC RESPONSE TO TF-HSA AFTER IMMUNIZATION WITH VACCINES CONTAINING TF ANTIGEN

		Reciprocal median ELISA titers				*Median dot blot immune stain*			
		IgM		*IgG*		*IgM*		*IgG*	
Vaccine[a]	*No. of mice*	*TF-HSA*	*PSM*	*TF-HSA*	*PSM*	*TF-HSA*	*PSM*	*TF-HSA*	*PSM*
None	5	20	20	0	0	0	0	0	0
PSM	5	20	40	0	0	0	0	0	0
PSM + CFA	5	40	40	0	320	0	0	1+	2+
PSM + Detox	5	40	40	0	40	0	0	0	1+
PSM + SAF-m	4	40	20	0	2560	0	0	0	2+
PSM + QS21	5	40	20	0	2560	0	0	0	2+
TF ceramide (c)	5	0		0		0		0	
TFc + R595	5	0		0		1+		0	
TFc + proteosomes	5	0		20		1+		1+	
TFc + CFA	5	40		0		1+		0	
TFc + SAF-m	5	0		0		1+		1+	
TFc + QS21	5	0		0		1+		0	
			Epi[c]		Epi		Epi		Epi
TF-KLH 3100/1[b]	5	40	10	80	0	2+	0	1+	1+
TF-KLH	20	0	0	20	0	1+	0	0+	1+
TF-KLH + CFA	10	40	0	40	0	2+	1+	1+	1+
TF-KLH + Detox	15	40	0	40	0	2+	0	1+	1+
TF-KLH + proteosomes	10	160	0	320	0	1+	0	1+	1+
TF-KLH + SAF-m	20	80	5	>10,000	0	2+	1+	3+	1+
TF-KLH + QS21 (30 μg)	5	20	10	10,000	0	2+	1+	3+	1+
TF-KLH + QS21 (10 μg)	20	160	20	>10,000	0	2+	1+	3+	1+
TF-KLH + QS21 (3 μg)	5	80	20	5,000	0	2+	1+	3+	1+

[a] Vaccines were administered sc to groups of 5 mice 3 times at 2–3 week intervals. Mice were bled before each vaccination and 2 weeks after the third vaccination.

[b] TF-KLH ratio 860:1 unless otherwise stated.

[c] Epi, epiglycanin.

Serologic response against KLH after vaccination

The serologic response against KLH was tested to determine whether the immunologic adjuvants resulting in the highest anti-TF serologic reactivity were the same as those resulting in the highest antiprotein titers. IgG antibodies were detected in all cases, but once again, the titers were highest with the use of SAF-m and QS21 (Table 2).

Delayed type hypersensitivity responses after vaccination

Footpad injections with TF-HSA were performed 2 weeks after the third immunization in all mice. DTH responses were not detected after immunization with any of the vaccines. To more closely approximate the DTH testing schedule followed by others, we immunized two additional groups of 5 mice with TF-KLH plus Detox and tested for DTH against HSA and TF-HSA 2 weeks after vaccines one and two, as well as vaccine three. No DTH was detected after the first immunization. DTH reactivity against HSA and TF-HSA was detected after the second and third immunizations. The median DTH response to HSA after the second and

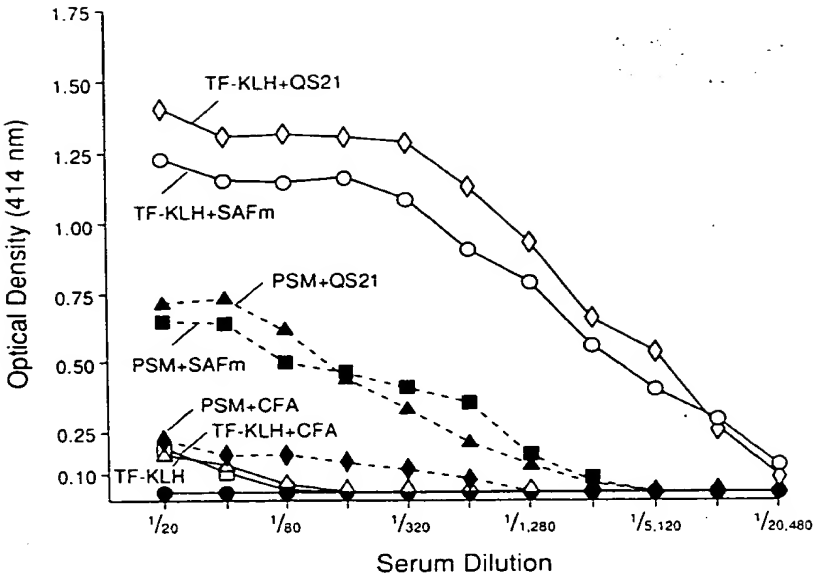

FIG. 1. IgG antibody levels detected by ELISA in mice receiving various TF-KLH vaccines (tested on TF-HSA target) or PSM vaccines (tested on PSM target). Each lane indicates results with serum from a different mouse. Each point represents the median optical density at that titer for all mice receiving the indicated vaccine. Serologic reactivity for mice immunized with PSM alone and tested on PSM or for all mice receiving PSM plus or minus adjuvant vaccines tested on TF-HSA was 0, as indicated by the closed circles.

third immunizations was 0.08 mm and 0.16 mm, respectively. The mean additional increase in footpad swelling after testing with TF-HSA compared to HSA was 0.11 mm and 0.14 mm after the second and third immunizations, respectively, at 48 h (reactions were present but smaller at 24 h). To further evaluate the specificity of this reaction, DTH tests with epiglycanin, desialoglycophorin, and glycophorin were performed 7–14 days after the TF-HSA tests. No reaction was seen. We conclude (1) that immunization with TF-KLH resulted in no DTH against TF-HSA and (2) that skin testing with TF-HSA itself results in DTH against HSA and additional DTH against a TF-HSA epitope that is not present on natural TF expressed on epiglycanin and desialoglycophorin. This epitope may contain aspects of both TF and HSA.

DISCUSSION

The purposes of this study were to (1) test whether PSM and synthetic TF disaccharide conjugates could induce immunity against TF antigen as it is naturally expressed in glycoproteins and mucins, (2) determine which form of the synthetic antigen was most immunogenic, that is, antigen conjugated to a hydrophobic group (ceramide) or antigen conjugated to an immunogenic protein (KLH), and (3) determine whether the use of immunologic adjuvants was capable of further augmenting this immunogenicity. The studies showed that synthetic TF disaccharide-KLH conjugate is capable of augmenting natural IgM antibody titers reactive with TF antigen expressed on desialoglycophorin (desialylated RBC) and on tumor mucins of murine and human origin. They also show that the immunologic adjuvants QS21 and SAF were capable of greatly augmenting the IgG antibody response against synthetic TF disaccharide, PSM, and KLH, but no adjuvant was able to augment the immunogenicity of TF antigen expressed on PSM or of TF disaccharide-ceramide. Finally, they show that unlike the induced IgM antibodies, the induced IgG antibodies are not reactive with epitopes expressed on such natural TF antigens as asialoglycophorin and tumor mucins.

Det ction of IgM Antibody In Sera of Mice Immunized with TF disaccharide-KLH Vaccines

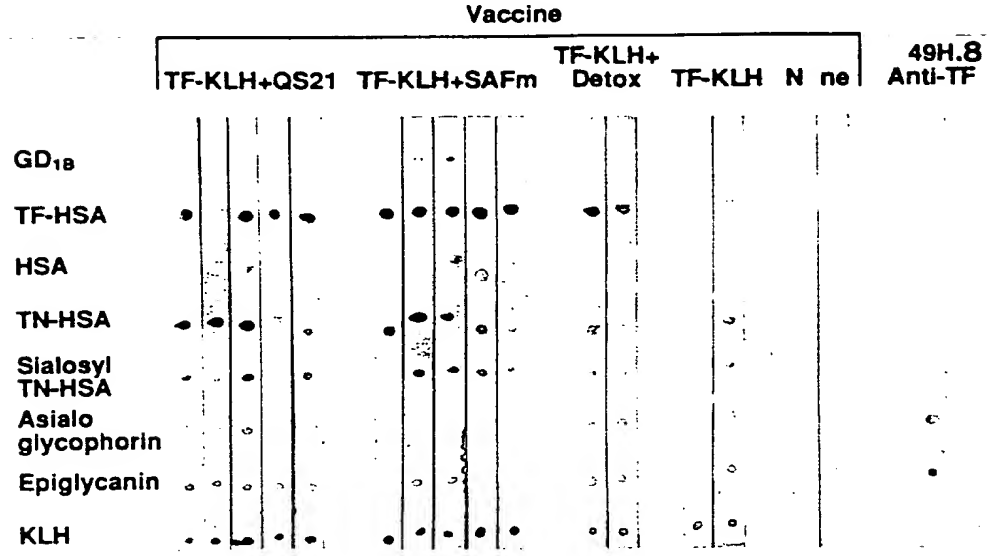

FIG. 2. Dot blot immune stain assay for IgM antibodies in sera of mice immunized with TF-KLH. Antigen standards were applied to silica gel strips and allowed to react with preimmunization (none) or peak titer postimmunization serum from a different mouse. Strips were developed with antimurine IgM antibody labeled with peroxidase. The vaccines used for immunization are indicated on the horizontal axis and the antigen spotted on all strips are indicated on the vertical axis. Reactions on the first TF-KLH plus QS21 strip indicate reactivity in the peak titer postimmunization serum from a mouse immunized with TF-KLH plus QS21 and are graded from top to bottom: ±, 3+, 1+, 3+, 2+, 1+, 2+, and 3+.

Failure of the IgG antibodies induced by synthetic TF-conjugates to recognize TF epitopes on natural glycoproteins and mucins was not entirely unexpected. We have immunized mice and melanoma patients with a series of synthetic ganglioside antigens, including GD3 lactone, GD3 gangliosidol, and GD3 amide,[22,23] as well as several O-acetyl GD3 products.[24] In each case, patients (and, in most cases, mice) identified artifactual synthetic epitopes that were not present on natural gangliosides to the exclusion of the epitopes that were present on the natural gangliosides, even though typing murine monoclonal antibodies recognized synthetic and natural gangliosides equally well. In this case, though murine anti-TF monoclonal antibody reacts well with both synthetic disaccharide conjugate and natural TF antigen (Fig. 2), the high titer IgG antibodies induced against the synthetic antigen failed to react with natural TF antigen. It is curious that natural low level IgM antibodies that showed increased titers against the disaccharide conjugate after vaccination were able to cross-react with TF antigen from natural sources. This may represent augmentation of the low affinity, germ line IgM antibody response often seen against carbohydrate antigens.[25] The altered specificity of the IgG antibody response against TF disaccharide may represent subversion of germ line specificity by somatic mutation of V region genes known to occur in the context of T cell help, as has been described after vaccination with phosphorylcholine-KLH conjugates.[26,27] It also may be that the epitope seen by these IgG antibodies includes the 2-carbon crotyl linker arm used to attach disaccharide to KLH and HSA and possibly a part of the proteins. Lack of reactivity of these IgG antibodies with TF-ceramide (which has no linker arm) favors this explanation.

Repeated skin testing for DTH was shown to induce DTH responses against HSA and significant additional DTH responses against TF-HSA. No DTH reactivity against natural sources of TF antigen could be demonstrated in these mice. When only a single skin test was performed after the third immunization, no

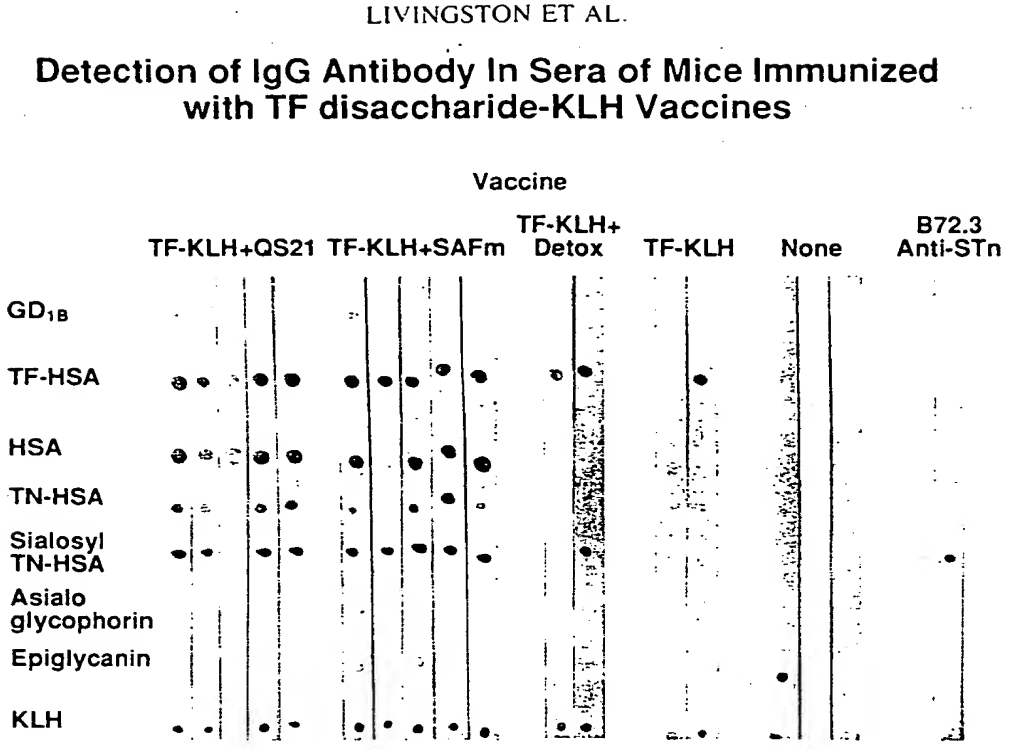

FIG. 3. Dot blot immune stain assay for IgG antibodies in sera from mice immunized with TF-KLH. Antigen standards were applied to silica gel strips and allowed to react with preimmunization (none) and peak titer postimmunization serum from a different mouse. Strips were developed with peroxidase-labeled antimurine IgG antibody. The vaccines used for immunization are indicated on the horizontal axis, and the antigen spotted on all strips is indicated on the vertical axis. Reactions on the first TF-KLH plus QS21 strip indicate reactivity in the peak titer postimmunization serum from a mouse immunized with TF-KLH plus QS21 are graded from top to bottom: 2+, 3+, 3+, 3+, 3+, 0, 1+, and 3+.

consistent DTH was seen against HSA or TF-HSA. These results suggest immunization by the skin testing procedure, as was initially described in the clinical setting with other antigens.[28] We previously have reported induction of TF-HSA DTH reactivity by immunization with TF-KLH[7] and are uncertain why no DTH was seen here. There were several major differences in the immunization and DTH testing procedures used:

TABLE 2. SEROLOGIC RESPONSE TO KLH AFTER IMMUNIZATION WITH VACCINES CONTAINING TF-KLH PLUS VARIOUS IMMUNOLOGIC ADJUVANTS

Vaccine[a]	*No. of mice*	*Median anti-KLH ELISA titer IgG*
None	5	1/40
QS21 alone	5	1/80
TF-KLH	10	1/160
TF-KLH + Detox	10	1/320
TF-KLH + CFA	10	1/640
TF-KLH + SAF-m	10	1/10240
TF-KLH + QS21	10	1/10240

[a] Vaccines were administered sc to groups of 5 mice 3 times at 2–3 week intervals. Mice were bled before each vaccination and 2 weeks after the third vaccination. TF-KLH ratio 860:1.

previously A/J × BALB/c F_1 mice were used (as opposed to C57B46 × BALB/c F_1), an immunization dose of 100 μg TF-KLH (as opposed to 25 μg) and a footpad injection dose of 50 μg of TF-HSA (as opposed to 5 μg) were used, and Ribi murine adjuvant containing trehalose dimycolate plus MPLA was used with TF-KLH (as opposed to Detox containing BCG cell wall skeletons and MPLA). These differences will be the focus of future experiments.

The use of immunogenic carrier proteins to augment the immunogenicity of small, poorly immunogenic haptens or other antigens dates back to the experiments of Landsteiner.[29] Potent T cell help is induced against the carrier protein, which permits B cell (and, in some cases, T cell) responses against the poorly immunogenic antigen to be greatly augmented and class switching and affinity maturation to occur. Whether the need for T cell help with these antigens is based on inability of antigen presenting cells to process the antigens as is assumed to be the case with carbohydrate antigens or T cell tolerance as is assumed to be the case with many self-antigens, the effect is the same. This approach, termed *conjugate vaccines*, has been used to induce IgG antibodies to capsular polysaccharide antigens of *Haemophilus influenzae* type B (and protection) in infants[30] and to induce IgG antibodies against a variety of other carbohydrate antigens in experimental animals.[31] We show here that this approach also applies to TF disaccharide-KLH conjugates and further that it is greatly augmented by the additional use of potent adjuvants. One of the reasons for using PSM as a source of TF antigen was the assumption that the xenogeneic mucin core protein might function as an immunogenic carrier. This clearly was not the case. The explanation for the lack of T cell help provided by PSM may be the intense glycosylation characteristic of mucins or the nature of the protein core itself. It also is possible that TF antigen expressed on PSM is antigenically distinct from TF antigen expressed on our other natural or synthetic products, though once again all react well with anti-TF MmAb 49H.8.[18]

The immunogenicity of PSM and the TF disaccharide and KLH components of these vaccines were augmented by the immunologic adjuvants CFA, Detox, SAF-m, and QS21. This was especially true for SAF-m and QS21. Of the three major modes of action for immunologic adjuvants (reviewed in reference 14), two, depot effects (generally produced by aluminum compounds or lipid emulsions) and macrophage stimulation (produced here by lipid A, MDP, CFA, and so on), are produced by most available adjuvants. The third mode of action, stimulation of B and T lymphocytes, shows a greater variation between adjuvants and is more difficult to define. BCG and one of its active components MDP are known to stimulate B and T lymphocytes, and lipid A is a mitogen for murine B cells. Whether it is these direct effects, the level of any given cytokine, or more likely the resulting changing mix of cytokines that are most responsible for adjuvant effects with each antigen is unknown. It also is not clear whether carbohydrate-protein conjugates are processed or have adjuvant requirements different from proteins or peptide-protein conjugates. Our results suggest that SAF-m and QS21 are highly effective adjuvants for inducing anti-TF disaccharide antibodies after immunization with TF-KLH and also that they induce the highest titer PSM and KLH antibodies. With regard to immunization with gangliosides (in the absence of a carrier protein), our experience has been the reverse. R595, proteosomes, and Detox all are more effective adjuvants than SAF-m or QS21 for induction of the ganglioside antibody response, which is predominantly IgM (unpublished observations). As the effects of immunologic adjuvants on antigen processing and presentation and on lymphocyte activation become better understood, it may be possible to select the optimal adjuvant for any antigen based on this knowledge. For now, however, the process is largely empirical. The immunologic adjuvants we have chosen for testing represent a cross-section of the many adjuvants that have been described. Several additional adjuvants are commercially available for use in experimental animals, and we also plan to test these. In addition, ongoing studies with super antigens[32] and heat shock proteins[33] may result in novel approaches for augmenting antigen presentation and lymphocyte activation, thereby circumventing or further augmenting the effect of currently available carrier proteins and adjuvants.

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